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(54) RECOMBINED DNA VIRUS VECTOR FOR INFECTION OF ANIMAL CELL

(57)Abstract:
PURPOSE: To obtain the recombined DNA virus vector capable of introducing a foreign gene into an animal cell in the form capable of being autonomously replicated in a wide range of animal cells.
CONSTITUTION: The recombined DNA virus vector for infecting the animal cell has a promoter, a recombinase gene and a poly A sequence. The recombined DNA virus vector for injecting the animal cell has two recombinase-recognizing sequences and a replication point-starting point acting on the animal cell, the promoter, the foreign gene and the poly A sequence placed between the two recombinase-recognizing sequences. The method for introducing the foreign gene into the cell comprises infecting the animal cell with the recombined DNA virus vector and subsequently autonomously replicating a foreign gene-expressing unit in the cell. And a method for genetically treating a genetic disease comprises introducing a human gene into the cell by the method for introducing the gene into the cell. The recombined DNA virus vector is useful for the therapy of the genetic diseases.

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* NOTICES *

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CLAIMS

[Claim(s)]
[Claim 1] The recombinant DNA virus vector for animal cell infection which has a promotor, a recon BINAZE gene, and a poly A array.
[Claim 2] The recombinant DNA virus vector according to claim 1 whose DNA virus vector is an adenovirus vector.
[Claim 3] The DNA virus vector according to claim 2 whose recon BINAZE gene is a gene of recon BINAZE Cre of the Escherichia coli P1 phage origin.
[Claim 4] The recombinant DNA virus vector for animal cell infection which has the replication origin and promotor who work by the animal cell in two recon BINAZE recognition sequence lists in the meantime, a foreign gene, and a poly A array.
[Claim 5] The recombinant DNA virus vector according to claim 4 whose DNA virus vector is an adenovirus vector.
[Claim 6] The recombinant DNA virus vector according to claim 5 in which the replication origin and promotor who work by the animal cell, the foreign gene, and the poly A array are carrying out orientation to this order from the upstream.
[Claim 7] The recombinant DNA virus vector according to claim 5 in which the replication origin and promotor who work by the foreign gene, the poly A array, and the animal cell are doing orientation to this order from the upstream.
[Claim 8] A recombinant DNA virus vector given in any 1 term of claim 4 to claim 7 which is the DNA array of loxP from which a recon BINAZE recognition sequence serves as a substrate of recon BINAZE Cre.
[Claim 9] A recombinant DNA virus vector given in any 1 term of claim 4 to claim 8 whose replication origin which works by the animal cell is the thing of the virus origin or the animal cell origin.
[Claim 10] The recombinant DNA virus vector according to claim 9 whose replication origin which works by the animal cell is that as which it is chosen out of the papovavirus, a Herpes virus, adenovirus, poxvirus, and the group which it becomes from the thing of the parvovirus origin.
[Claim 11] A recombinant DNA virus vector given in any 1 term of claim 1 a promotor and whose poly A are the hybrid promoters (CAG promotor) which consist of the splicing acceptor and poly A array of a cytomegalovirus enhancer, a fowl beta-actin promotor, and a rabbit beta globin - claim 10.
[Claim 12] The recombinant DNA virus vector for animal cell infection which has a promotor, a recon BINAZE gene, and a poly A array, The replication origin which works by the animal cell in a list two recon BINAZE recognition sequences and in the meantime, The recombinant DNA virus vector for animal cell infection which has a promotor, a foreign gene, and a poly A array is infected with an animal cell. The intracellular introducing method of the foreign gene by starting the replication origin and promotor who work by the animal cell which consists between two recon BINAZE recognition sequences, a foreign gene, and a poly A array as a cyclic molecule, and making it replicate autonomously by intracellular.
[Claim 13] The intracellular introducing method of a foreign gene according to claim 12 each DNA virus vector is an adenovirus vector.
[Claim 14] The intracellular introducing method of the human gene characterized by using the intracellular introducing method according to claim 12 or 13 on the occasion of gene therapy.

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DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Industrial Application] This invention relates to the recombinant DNA virus vector for animal cell infection. It is related with the use to the intracellular introducing method and gene therapy of the foreign gene using the recombinant DNA virus vector which includes in more detail the DNA array which carries out the code of the recognition sequence of a recon BINAZE gene or this recon BINAZE, and this ** KUTA.
[0002]
[Description of the Prior Art] Although the retrovirus was well used as a virus vector of transgenics until now, this virus has a problem from the viewpoint of that safety especially in gene therapy by being included in the chromosome of that it can introduce only into the divided cell, or a host cell, and it is thought that that application range is restricted. An adenovirus vector also has further that installation [about 100% of] effectiveness is show by various animal culture tissues and not have the device of positive chromosome inclusion unlike a retrovirus, and the advantage that transgenics can be carry out also in the cell of a resting phase, and it is think that the application range as a vector of a foreign gene installation experiment is very wide, and will be establish as one of the main techniques of gene therapy in the near future.
[0003] Use of an adenovirus vector is spreading quickly as one of the gene therapy techniques in respect of manifestation research in the cell which specialized in altitude, such as a nervous system. the gene which suffered a loss directly to the viable cell which is bearing the function is compensated by medicating directly the organization which is already built and is functioning as a gene therapy technique -- being the so-called -- Research is energetically advanced as the approach of in vivo gene therapy. By cystic fibrosis, five groups can actually accept the experiment therapy to a patient in the U.S., and it already came to inquire actively to a muscular dystrophy, familial hypercholesterolemia, a brain tumor, etc. on the other hand, the cell in which transgenics is possible for an adenovirus vector and it specialized also to the cell of a resting phase -- the transgenics experiment to primary culture or an animal individual attracts attention as the transgenics approach to a nervous system especially. As mentioned above, especially the application to gene therapy is expected from the gene expression not only by many differentiation containing a nervous system and the gene therapy installation to an undifferentiated cell but direct impregnation and administration for an animal individual being possible for an adenovirus vector.
[0004] However, since adenovirus does not have the device of positive chromosome inclusion unlike a retrovirus its manifestation is temporary. From one - two weeks, even if the period is long, it is about two months. Therefore, when it is necessary to make a curative effect continue, the manifestation by repeat administration needs to be continued. However, in repeat administration, we are anxious about reduction of the curative effect by the appearance of an antibody. Therefore, the purpose of this invention has the foreign gene introduced by the adenovirus vector in the animal cell in building the system of the recombination adenovirus vector which can be changed into the form which can be replicated autonomously by intracellular, and it is further to provide gene therapies with this system.
[0005]
[Means for Solving the Problem] this invention persons inquire wholeheartedly, in order to solve the above-mentioned problem. Then, as a DNA virus vector for animal cell infection While cyclic-molecule-izing the gene expression unit containing the foreign gene introduced into intracellular using the adenovirus vector by using recon BINAZE and its recognition sequence By giving a replication origin there, it succeeded in changing the gene expression unit containing the foreign gene introduced into intracellular using the adenovirus vector into th

form which can be replicated autonomously.
[0006] Here, recon BINAZE is a specific DNA recombination enzyme, the specific DNA array which consists of dozens bases is recognized here, and all the processes of exchange of cutting and the chain of DNA and association are performed between this array. Then, if the recombination adenovirus vector which discovers this enzyme, and the recombination adenovirus vector which has two copies for this recognition sequence in the same direction are produced and the sympathy stain of both is carried out to a cell, reconstruction between two recognition sequences will break out by discovered recon BINAZE, and the pinched part will be started as a cyclic molecule. Therefore, if the manifestation unit and the replication origin are included in this part, a cyclic molecule is reproduced within a nucleus, is permanently maintained in intracellular, and can continue the manifestation of a foreign gene. Therefore, if the system of such a recombination adenovirus vector is used for gene therapy, it will become possible by 1-time administration to make a prolonged curative effect maintain. Based on this knowledge, this invention advances research further and comes to be completed.
[0007] Namely, summary of this invention (1) The recombinant DNA virus vector for animal cell infection which has a promotor, a recon BINAZE gene, and a poly A array, (2) The recombinant DNA virus vector of the aforementioned (1) publication whose DNA virus vector is an adenovirus vector, (3) The DNA virus vector of the aforementioned (2) publication whose recon BINAZE gene is a gene of recon BINAZE Cre of the Escherichia coli P1 phage origin, (4) The replication origin which works by the animal cell in two recon BINAZE recognition sequence lists in the meantime, The recombinant DNA virus vector for animal cell infection which has a promotor, a foreign gene, and a poly A array, (5) The recombinant DNA virus vector of the aforementioned (4) publication whose DNA virus vector is an adenovirus vector, (6) The recombinant DNA virus vector of the aforementioned (5) publication in which the replication origin and promotor who work by the animal cell, the foreign gene, and the poly A array are carrying out orientation to this order from the upstream, (7) The recombinant DNA virus vector of the aforementioned (5) publication in which the replication origin and promotor who work by the foreign gene, the poly A array, and the animal cell are doing orientation to this order from the upstream, (8) A recombinant DNA virus vector given in any 1 term of the above (4) to the above (7) which is the DNA array of loxP from which a recon BINAZE recognition sequence serves as a substrate of recon BINAZE Cre (9) A recombinant DNA virus vector given in any 1 term of the above (4) to the above (8) whose replication origir which works by the animal cell is the thing of the virus origin or the animal cell origin, (10) The replication origin which works by the animal cell The papovavirus, a Herpes virus, Adenovirus, poxvirus, and the recombinant DNA virus vector of the aforementioned (9) publication which is what is chosen from the group which consists of a thing of the parvovirus origin, (11) A promotor and poly A Cytomegalovirus enhancer,

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EXAMPLE

[Example] Hereafter, although an example and the example of reference explain this invention in more detail, this invention is not limited at all by these examples etc. In addition, especially many actuation of dealing with the phage in an example, a plasmid, DNA, various enzymes, Escherichia coli, a cultured cell, etc. was performed to "the volumes Molecular Cloning and on A Laboratory Manual.T.Maniatis, the 2nd edition (1989), and Cold Spring Harbor Laboratory" according to the approach of a publication, unless it refused. Moreover, a DNA restriction enzyme and repair enzyme are TAKARA SHUZO and New. England It purchased from Biolabs (NEB), Stratagene, or Boehringer, and was used according to manufacturer instructions.
[0037] An example 1< recon BINAZE Cre gene And Escherichia coli P1 phage DNA (ATCC11303-B23) containing the production ** recon BINAZE Cre gene of the cassette cosmid for production >(1) recon BINAZE Cre gene expression of the recombination adenovirus vector which has a CAG promotor is made into a template. The following (array number as a 5'-primer : the oligonucleotide of 1) The following (array number as a 3'-primer : the oligonucleotide of 2) VentR of the product made from NEB as heat-resistant polymerase It used, the PCR reaction was performed on condition that the following, the product was applied to agarose gel electrophoresis, the band of about 1 kb was cut down, and the DNA fragment of about 1 kb containing a recon BINAZE Cre gene was obtained.
5'-CGT CTGCAG TGCA TCATGA GTAATTTACTGACCGTACACCAAAATTTGCCTGC-3' PstI BspHI3'-GACCTTCTACCGCTAATCGGTAAT TCGCGAGATCT CGG-5' Aor51 HI;XbaI (an underline part is the recognition site of a restriction enzyme.)
[0038] PCR reaction condition buffer solution: MgSO4 of KCl of 10mM, Tris-HCl (pH8.8) of 20mM, 2 (NH4) SO4 of 10mM, and 2mM, 0.1% of Triton X-100 (the buffer solution of NEB attachment is used)
Heat-resistant polymerase: 2 unit dNTP: 400microM primer: 1microMP1 phage DNA: Two 1ng(s) chain dissociation temperature: Annealing temperature during 1.5 minutes: 1.5-minute expanding reaction temperature: 2.0-minute reaction cycle: 20 times [0039] It collects, after carrying out coincidence digestion of this fragment and pUC19 (TAKARA SHUZO make) with restriction enzymes PstI (TAKARA SHUZO make) and XbaI (TAKARA SHUZO make), respectively, it mixes so that a mole ratio may be set to about 3:1, and T4 DNA ligase (TAKARA SHUZO make) is used, and it is ligation. It reacted. Furthermore, the transformation of 109 shares (ATCC53323) of Escherichia coli JM was carried out using this cocktail. The transformant was gathered from LB agar plate which added ampicillin (100microg/(ml)), and the plasmid pUCCre containing a recon BINAZE Cre gene was obtained.
[0040] Next, coincidence digestion of the 1micro of the things g and pUCCre which cut a cell technology and cassette cosmid pAdex1CAwt which contains in 13,760-763 (1994) the CAG promotor who prepared by the approach of a publication by SwaI was carried out by PstI and XbaI, and 0.1micro of fragments g of about 1 kb which graduated both ends with the Klenow enzyme (TAKARA SHUZO make) further, and was obtained was mixed. They are pCAGGS (22 pages - of one line 25 pages 13 pages - of 20 lines 20 pages JP,3-168087,A and 14 lines and, six lines) to the restriction enzymes SalI and HindIII with which the CAG promotor is indicated as a high expression vector here at JP,3-168087,A, and the preparation is indicated at this official report. By starting, it can carry out and can use for this invention.
[0041] ** Next, ethanol was added to mixed liquor and cosmid was settled. Settlings were acquired according to centrifugal separation and it dissolved in the 5 time diluent of the solution (TE) which added EDTA of 1mM to the 10mM tris-hydrochloric acid (pH7.5).
** ATP and T4 DNA ligase were added in the ligase reaction buffer, and the obtained cosmid was combined by the last capacity I of 7micro overnight. Subsequently, thermal inactivation of the ligase was carried out 70-

degree-C 10 minutes after adding sterilized water and the SwaI reaction buffer and being referred to as 48micro Under the present circumstances, unlike PURAZUMIDO, by cosmid, it is not annular and the macromolecule combined with the straight chain-like tandem is packed efficiently.
[0042] ** SwaI (product made from Boehringer) of 2microl was added, and it cut at 25 degrees C for 1 hour. Since a SwaI recognition sequence will be reproduced if recombined without cassette cosmid adding a manifestation unit, the semantics which performs SwaI cutting is for re-cutting the cosmid in which a manifestation unit is not included at this step, and carrying out [do not make a colony and] it. This approach is the powerful approach of choosing only cassette cosmid with an insertion.
** a conventional method (Molecular Cloning vol.3 E.34) -- following -- the phenol extract of cassette cosmid, and centrifugal separation -- subsequently gel filtration was performed.
** SwaI cutting was performed again. Namely, SwaI of 5microl was added during the SwaI reaction buffer, and it cut at 25 degrees C for 2 hours. The reason is as above-mentioned.
[0043] ** The Inn vitro packaging was performed about 1microl of the obtained cosmid. That is, the G back XL (product made from Stratagene) who is a lambda Inn vitro packaging kit was used on 1/4 scale, and the remainder froze him at -80 degrees C. Since the package effectiveness of the cosmid of 42 or less kbs is low, the G back XL can choose the cosmid which the insertion entered and became large to some extent. In this experiment, when gathering ten colonies, most includes the insertion and was able to obtain the clone of the target sense (facing the left) easily. Cosmid treated and it carried out about the direction according to the conventional method (experimental medicine besides Saito Izumi: 7:183-187, 1989).
[0044] ** The cosmid by which packaging was carried out was infected with DH1 (ATCC33849). That is, 1/200 amount, 1/20 amount, 1/2 amount, and the remaining whole quantity were inoculated into Ap+ agar plate (ampicillin addition) of three sheets, and 5ml Ap+LB (pool), respectively, and it cultivated overnight. miniprepDNA of pool was extracted and prepared, and the rate was investigated although the insertion entered by all enzyme cutting. The colony was taken the whole agar the whole round head, it is 1.5ml Ap+LB, and was cultivated overnight and prepared miniprepDNA.
** Next, the sense and structure of a manifestation unit were checked by restriction enzyme cutting. In addition using NruI and a ligase, although the manifestation unit was included, the plasmid which carried out deletion of most adenovirus DNA was produced, DNA was prepared, and the last check of cDNA cloning was carried out.
[0045] (2) As preparation ** adenovirus DNA of adenovirus DNA-protein complex (Ad5 dlX DNA-TPC), it is Ad5 dlX (I.Saito et al., J.Virology, vol.54, and 711-719 (1985)) was used. Ad5 It cultivated by infecting dlX with a HeLa cell (Roux 10 duty). That is, 0.2 ml/Roux infection of the virus liquid (- 109 PFU/ml) of Ad5-dlX was carried out, in 1500rpm and 5 minutes, centrifugal separation of the cell which peeled three days after was carried out, and they were collected. Since most adenovirus particles are in [instead of in MEDIUMU] the nucleus of a cell, it has the advantage which can refine a virus from an infected cell. (The following actuation was performed in un-sterile.)
[0046] ** The obtained cell was suspended in 20ml of Tris-HCl (pH8.0) of 10mM(s), the cell was crushed in 200 W or 2 minutes (30 second x4) using seal mold SONIKETA, and a virus was made to emit from intracellular. Although five freeze thawing is sufficient if it is 5ml or less making a virus emit from intracellular, by the capacity beyond it, SONIKETA is convenient. However, a seal mold (thing with an exclusive cup) is surely used. The usual throwing-in mold is dangerous also in a safety cabinet.
[0047] ** Ultracentrifuge after removing precipitation for the obtained debris according to centrifugal separation (10krpm, 10 minutes) The 15ml cesium chloride solution (specific gravity 1.43) was put into SW28 tube, multistor [of the supernatant liquid] was carried out on it, and concentration by cushion centrifugal (25krpm, 1 hour, 4 degrees C) was performed.
** The virus layer directly under an interface was moved to SW50.1 tube. The virus layer directly under an interface could usually be viewed, and extracted a virus layer and 5ml of its lower layer cesium chloride. The cesium chloride solution (specific gravity 1.34) was filled to coincidence one more. These were applied to ultracentrifuge at 35krpm and 4 degrees C overnight. Subsequently, the band of a white virus is isolated preparatively and it put on the tube which was already able to do inclination again. Furthermore, it applied to 35krpm and 4-degree-C 4-hour or more ultracentrifuge.
[0048] ** The band of a white virus was isolated preparatively, it mixed at equivalent 8M guanidine hydrochloride and an equivalent room temperature, 4M guanidine hydrochloride saturation cesium chloride was added, and it filled in VTi65 tube. Particle protein was dissociated in response to denaturation, and DNA-TPC was emitted by 4M guanidine hydrochloride. Since the approach of removing later was not established, ethidium Promid was not

able to be used.
[0049] ** the existence of DNA was checked by covering the above-mentioned tube over ultracentrifuge at 55krpm and 15 degrees C overnight, and it carrying out fractionation 0.2ml at a time, and mixing with 20micro of 1microg [/ml] ethidium bromide water solutions I, and carrying out fluorescent staining of every [the 1microl]. Two to 3 fractions containing DNA were collected.
** It dialyzed to 500ml TE overnight (2 times), and saved at -80 degrees C. in this way, obtained Ad5dlX the amount of DNA-TPC -- OD260 from -- it computed like the usual DNA.
** Obtained Ad5dlX After cutting DNA-TPC by EcoT22I of an amount enough for 2 hours for recombination adenovirus creation of the 3rd step, it was saved at -80 degrees C.
[0050] In addition, although DNA-TPC could do cutting by the restriction enzyme, dialysis, and gel filtration, electrophoresis, phenol processing, and ethanol precipitation were not made. Since there was cesium chloride balance centrifugal, if possible, the condensing method was maintained at the thick condition. About 300microabout g DNA-TPC was able to be obtained from the infected cell of 10Roux(es).
** Isolate a part preparatively and it is BPB for migration. After 10microl Adding buffer, added the proteinase K (10mg/(ml)) of 1microl, it was made to react for 10 minutes at 37 degrees C, and end protein was digested. The phenol extract was carried out, supernatant liquid was separated by agarose gel electrophoresis, and full cutting was checked. After removing the restriction enzyme buffer in EcoT22I cutting DNA-TPC by centrifugal gel filtration, it poured distributively and saved at -80 degrees C.
[0051] (3) It prepared each 6cm of 293 cells cultivated by separation of a recombination virus, and the production ** 10%FCS addition DME of high potency virus liquid, and one 10cm petri dish.
** pAdex1w incorporating a manifestation unit Ad5dlX cut by 8microg (3-9microg is suitable) and EcoT22I of DNA 1microg of DNA-TPC was mixed and transfection was performed on one 6cm petri dish by the calcium phosphate method using the cel FEKUTO (Pharmacia manufacture) kit. Mixed liquor was dropped from on MEDIUMU of 6cm petri dish, and culture was continued. night culture (about 16 hours) -- carrying out -- during the morning -- culture medium -- exchanging -- three evening and collagen coat 96 holes (an undiluted solution 10 time dilution, and 100 time dilution) -- the 5%FCS addition DME -- using -- each -- it rewound by 0.1ml per well. For two dilution, 293 cells of 10cm petri dish were mixed every [3 / 1/], and were scattered so that the number of cells might not be greatly different with each plate.
[0052] ** three - four days, and eight - ten days after -- each -- the 10%FCS addition DME of 50microl was added to the well. When 293 cells became thin, it added a little early. The well to which a virus increased and the cell became extinct appeared during seven - the 15th. Whenever the cell of a well became extinct completely, it moved to 1.5ml tube which sterilized culture medium (every dead cell) with sterilization Pasteur pipette in sterile and quick freezing was carried out with dry ice, and it saved at -80 degrees C.
** The judgment was ended in 15 - 18 days. About ten culture medium tubes collected from the well from which the cell died comparatively late were chosen, and it saved at -80 degrees C after six freeze thawing by using as primary virus liquid (first seed) the supernatant liquid obtained by carrying out centrifugal for 5krpm 10 minutes. The well to which virus multiplication happened a little early is because the possibility of the mixed infection of two or more viral strains is high.
[0053] ** 293 cells were prepared for 24 hole plate, and it added FCS-DME (0.4ml / well) and 10micro of two wells of primary virus liquid I at a time 5%, respectively.
** if a cell will become extinct completely in about three days -- one well -- primary virus liquid production -- the same -- 6 times of freeze thawing, and centrifugal -- supernatant liquid -- obtaining -- this -- secondary virus liquid (second seed) ** -- it carried out and saved at -80 degrees C. The potency of secondary virus liqui was 107 - 108 PFU/ml extent. The at-long-intervals alignment of the cell into which other one wells became extinct was carried out by 5krpm(s) for 5 minutes, supernatant liquid was thrown away, and only the cell was saved at -80 degrees C (cel pack). When the cel packs of ten kinds of viral strains gathered, all DNA of an infected cell was extracted by the following approaches. In a cel pack, they are TNE for cell DNA of 400microl (50 mM Tris-HCl pH 7.5, 100mM NaCl, 10mMEDTA), and proteinaseK (10mg/(ml)) of 4microl. And 10%SDS of 4microl was added.
[0054] ** After processing at 50 degrees C for 1 hour, the nucleic acid subsequently obtained by ethanol precipitation was melted two phenol chloroform extractions and two chloroform extractions to TE of 50microl which contains RNase ml 20microg /. It cut by XhoI which is the enzyme which contains CG in a recognition sequence in the enzyme which cuts a manifestation unit for the 15microl, and with XhoI cutting of a manifestation cosmid cassette, electrophoresis was performed by agarose gel with a die length of about 15cm

overnight, and the pattern was compared. The band from the cutting point in a manifestation unit to the left end of an adenovirus genome chose what has appeared correctly. Moreover, since there was possibility of mixing witl a virus with deletion, the clone which can see [which cannot be explained] thinly was discarded. Since it increases to 10,000 copies per cell, Adenovirus DNA can extract all DNA together with Cell DNA, and can see the band of a viral DNA by restriction enzyme cutting. Since the enzyme which contains CG in a recognition sequence like XhoI does not cut Cell DNA, its pattern is legible. When using enzymes other than this, Lycium chinense was required for control in the non-infected 293 cell DNA. (The band of the reiterative sequence origin of a human cell appeared) .

[0055] ** 150cm2 which carried out the collagen coat of the 0.1ml of the secondary virus liquid of a viral strain to have been identified by XhoI cutting You made it infected to 293 cells of a bottle (a culture medium is 25ml). When the cell became extinct three days after, the every dead cellml [25ml] culture medium was crushed in sterile in closed mold SONIKETA 200w horsepower output 2 minutes (30 seconds x 4 times), and a virus was separated. Centrifugal was carried out for 10 minutes at 3krpm(s) and 4 degrees C, precipitation was removed, it poured distributively in each tube for 5ml freezing 13 [2ml], quick freezing was carried out with dry ice, it saved at -80 degrees C, and 3rd virus liquid was prepared. 3rd virus liquid was the liquid containing the recombination adenovirus of this invention, and was the thing of a high potency of 109 PFU/ml extent. In addition, the enzyme cutting pattern of the viral DNA which was infected with 293 cell 1 well of 24 hole plate, and increased 5micro of 3rd virus liquid I was checked by the above-mentioned approach. Since the deletion virus of growth already slightly intermingled in the phase of secondary virus liquid was early and it might be visible supposing it was suspected that it is mixture with a deletion virus or a parent virus, all 3rd seeds were discarded, and it redid anew from another secondary virus liquid, or the target virus was purified by limiting dilution from the primary virus liquid.

[0056] The recombination adenovirus of example <simple potency measuring method of recombination adenovirus of this invention> this invention of reference can be measured simple by the following approaches.

** It prepares 293 cells each one 10cm petri dish. Phase dilution of the recombination adenovirus liquid (3rd virus liquid) is carried out to 10-1 to 10-4 using the FCS addition DME 5%. For example, 0.9mlDME(s)+0.1ml virus liquid. All chips are changed.

** Put the FCS addition DME into all the wells of one collagen coat 96 **** 5% with an every [l] of 50micro. The recombination virus diluted to 10-4 is added to eye the 1st train every [25micro / l]. 25microl is moved to the well of eye two trains using the ** multichannel pipet 8 wells. The same actuation is repeated to 11 trains below, and the last 25microl is thrown away. It is 3n as a result. A phase dilution train is producible to 311x10-4. Eye 12 trains is taken as control of an uninfected cell. The chip used at this time is changed to whenever [that].

[0057] In the loxP array of 2< 2 examples, and a list in the meantime The replication origin of SV40, A CAG promotor, And a hepatitis B virus surface antigen (HBs) the plasmid pHBVadr4 (Fujiyama et.al. --) containing production ** HBs cDNA of the cassette cosmid for a production >(1) hepatitis-B-virus surface antigen (HBs) manifestation of the recombination adenovirus vector which it has Nucleic Acids Res., 11, and 4601- after carrying out coincidence digestion of 4610 and 1983 by restriction enzyme Psp1406I and XhoI, both ends were further graduated with the Klenow enzyme, and agarose gel electrophoresis recovered the fragment of 710bp(s).

[0058] ** The following actuation was performed in order to obtain the manifestation unit for making HBscDNA discover under a CAG promotor's control. Plasmid pCAGGS including a CAG promotor (Niwa et.al.Gene, Vol.108, P193-200) pCAWG obtained by inserting a SwaI linker in a cloning part was cut by SwaI, and alkaline-phosphatase processing was performed. Next, the fragment obtained by this and I was mixed by the mole ratio 1:3 [about], the T4 DNA ligase reaction was performed, and the transformation of the Escherichia coli DHI stock (ATCC33849) was carried out by the cocktail. The transformant was gathered from LB agar plate which added ampicillin, and plasmid pCAG-HBs by which the fragment was inserted in the direction which HBscDNA discovers under a CAG promotor's control was obtained.

[0059] ** The following actuation was performed in order to obtain the DNA fragment containing the replication origin of a HBs manifestation unit and SV40. After carrying out coincidence digestion of pCAG-HBs with restriction enzymes SapI and SalI and graduating both ends with a Klenow enzyme further, agarose gel electrophoresis recovered the fragment of 3.6kbs. After the restriction enzyme SmaI cut, pUC18 (TAKARA SHUZO make) which performed alkaline-phosphatase processing, and a 3.6kb fragment were mixed by the mole ratio 1:3 [about], the ligation reaction was performed, and plasmid pUC18CAHBsS made into the purpose was obtained.

[0060] ** The following actuation was performed in order to add a loxP part to the both ends of the DNA fragment which contains the replication origin of a HBs manifestation unit and SV40 next. After cutting pUC119 (TAKARA SHUZO make) by restriction enzyme Ecl136II and performing alkaline-phosphatase processing, when it has a MluI part and a XhoI part at the end and this connected with it, plasmid pULL2r in which the ligation reaction with the following synthetic DNA fragment (array number: 3) including the loxP array currently designed so that a NruI part may be produced was performed, and these two synthetic DNA fragments were inserted was obtained.
5'-CGAACGCGTATAACTTCGTATAGCATACATTATACGAAGTTATCTCGAGTCG-3'3'-GCTTGCGCATATTGAAGCATATCGTATGTAATATGCTTCAATAGAGCTCAGC-5' (the array of an underline part is a loxP part.)
After carrying out coincidence digestion of the pUC18CAHBsS obtained by above ** by the restriction enzyme SalI and Ecl136II and graduating both ends with a Klenow enzyme further, agarose gel electrophoresis recovered the fragment of 3.6kbs. After the restriction enzyme's NruI having cut pULL2r and performing alkaline-phosphatase processing, plasmid pULCA-HBsS which performs a ligation reaction with a 3.6kb fragment, and is made into the purpose was obtained.
[0061] ** In order to obtain the recombination cosmid containing the fragment which has a loxP part to the both ends of the DNA fragment containing the replication origin of a HBs manifestation unit and SV40, both the following DNA was prepared.
(a) Fragment 0.3microg of 3.7kbs collected by agarose gel electrophoresis after carrying out coincidence digestion of pULCA-HBsS with restriction enzymes SmaI and EcoRI and graduating both ends with a Klenow enzyme further.
(b) pAdexlcw(cell technology, 13,760-763, (1994)) 1microg cut with the restriction enzyme SwaI.
Both were mixed and the recombination cosmid made into the purpose by the completely same actuation as (1) ** of an example 1 - ** was obtained. By the still more nearly same actuation as (2) - (3) of an example 1, the recombination adenovirus vector AdexILCAHBsSL which has the replication origin, CAG promotor, and hepatitis B virus surface antigen (HBs) of SV40 in two target loxP arrays and a list in the meantime was obtained.
[0062] Example 3 (infection experiment)
COS-1 cell or valve flow coefficient-1 cell -- 6cm petri dish -- mostly, a base is cultivated until it becomes like a wrap. Each of the adenovirus vector obtained in the example 1 and the example 2 was made to adsorb for 1 hour according to the protocol following by m.o.i.=5. Three days after, HARVEST was carried out and Southern analysis was carried out. That is, if the recombination adenovirus vector AdexILCAHBsSL has at least the HindIII cutting section of about 6.0 kbs and is cut by recon BINAZE Cre by the loxP part, it will serve as a cyclic molecule of 3.5kbs. Since this cyclic molecule had at least the one HindIII cutting section, it processed DNA which carried out after [infection] HARVEST by HindIII, applied it to potential migration, and performed Southern blotting. HBs fragmentation (710bp) was used for the probe. The result is shown in drawing 1 .
[0063] the line of 3.5kbs produced by HindIII cutting of a cyclic molecule only when you make it infected in the combination of the adenovirus vector obtained in the example 1 and the example 2 so that clearly from drawing 1 -- DNA was observed (a lane 4 and lane 8). Moreover, when infecting the adenovirus vector obtained in the example 2, and the adenovirus vector which does not have a recon BINAZE Cre gene (a lane 3 and lane 7), the band of 3.5kb is not accepted but only the band of 6.0kbs which it is started by HindIII and produced from AdexILCAHBsSL is accepted.
[0064] Furthermore, the comparison of the concentration of the band of a lane 7 and a lane 8 shows that the cyclic molecule was reproduced about 40 times by COS-1 intracellular. On the other hand, since the concentration of the band of a lane 3 and a lane 4 is almost comparable, in valve flow coefficient-1 intracellular, it turns out that a cyclic molecule is not reproduced. As for this, the replication origin of the SV40 origin agrees with the fact which is not committed in valve flow coefficient-1 intracellular. the above result is of-evidence attachment ** clearly about the part pinched by two recon BINAZE recognition sequences being started by intracellular, and forming a cyclic molecule, and replicating autonomously by intracellular by making it infected with an animal cell using the combination of the adenovirus vector of this invention obtained in the example 1 and the example 2.
[0065] In addition, it is expedient to conduct the above-mentioned infection experiment as follows. When the blood serum of a culture medium is not FCS (for example, CS), 2 times is washed for a cultured cell by the culture medium of a non-blood serum, and a culture medium is removed. Virus liquid (it dilutes with the culture medium of a non-blood serum or FCS addition) is added to extent to which a cell side does not get dry during

the following actuation. It is more practical to leave intentionally 100-200micro culture medium although it is about l, before adding virus liquid for a while with 50-70microl and 10cm petri dish in 30-40microl and 24 holes ii 96 holes, to add virus liquid, and to make it a capacity of this amount. It goes to a cell uniformly and virus liquid made to cross to it by shaking a plate in a cycle of several seconds like seesaw several times. This actuation is performed 3 times every 20 minutes. During this period and a cell are CO2. It sets to incubation. After the 3rd actuation finishes, culture medium is usually cultivated like an amount, in addition usual (1 hour after infection). Even if infection time amount is 1 hour long, it is usually enough in about 2 hours.

[Translation done.]

* NOTICES *

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2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

EFFECT OF THE INVENTION

[Effect of the Invention] By this invention, the recombinant DNA virus vector which can introduce a foreign gene into an animal cell in an extensive animal cell in the form which can be replicated autonomously can be offered. Moreover, this invention offers the simple manufacture approach of this recombinant DNA virus vector. Especially the recombination adenovirus vector of this invention is useful for the therapy of a hereditary disease.
[0067]
[Layout Table]
array number: -- die-length [of one array]: -- mold [of 53 arrays]: -- number [of nucleic-acid chains]: -- single strand topology: -- a nucleic acid (it is DNA of the arbitration containing Genomic DNA a part) besides class: of a straight chain-like array
hypothetical sequence: -- YES antisense: -- NO origin: -- approach:S which determined the description description of an Escherichia coli P1 phage DNA array Array CGTCTGCAGT GCATCATGAG TAATTTACTG ACCGTACACC AAAATTTGCC TGC 53 [0068] array number: -- die-length [of two arrays]: -- mold [of 38 arrays]: -- number [of nucleic-acid chains]: -- single strand topology: -- a nucleic acid (it is DNA of the arbitration containing Genomic DNA a part) besides class: of a straight chain-like array
hypothetical sequence: -- YES antisense: -- NO origin: -- approach:S which determined the description description of an Escherichia coli P1 phage DNA array Array GGCTCTAGAG CGCTTAATGG CTAATCGCCA TCTTCCAG 38 [0069] array number: -- die-length [of three arrays]: -- mold [of 52 arrays]: -- number [of nucleic-acid chains]: -- double strand topology: -- a nucleic acid (it is DNA of the arbitration containing Genomic DNA a part) besides class: of a straight chain-like array
hypothetical sequence: -- YES antisense: -- NO origin: -- approach:S which determined the description description of an Escherichia coli P1 phage DNA array Array CGAACGCGTA TAACTTCGTA TAGCATACAT TATACGAAGT TATCTCGAGT CG 52

[Translation done.]

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] Drawing 1 is drawing showing the result which processed back-collected DNA which was infected with COS-1 cell or valve flow coefficient-1 cell using the combination of various kinds of recombination adenovirus vectors by HindIII, and carried out fractionation by electrophoresis, and which was analyzed by Southern blotting.
[Description of Notations]
M Marker
1 Object Division Which Added Only Culture Medium into Valve Flow Coefficient-1 Cell
2 Division Incorporating Adenovirus Vector Which is Made to Carry Out Deletion of the E3, E1A, and E1 Area B to Valve Flow Coefficient-1 Cell, and Has Not Included Foreign Gene in it, Recon BINAZE Cre Gene Prepared in the Example 1, and CAG Promotor with which it Rearranged and Adenovirus Vector was Infected
3 Division with which Adenovirus Vector Which Valve Flow Coefficient-1 Cell is Made to Carry Out Deletion of the E3, E1A, and E1 Area B to Adex1LCAHBsSL (what was Prepared in the Example 2), and Has Not Included Foreign Gene in it was Infected
4 Division Which Included Adex1LCAHBsSL (what was Prepared in the Example 2), Recon BINAZE Cre Gene Prepared in the Example 1, and CAG Promotor in Valve Flow Coefficient-1 Cell and with which it Rearranged and Adenovirus Vector was Infected
5 Division Which Performed the Same Processing as 1 to COS-1 Cell
6 Division Which Performed the Same Processing as 2 to COS-1 Cell
7 Division Which Performed the Same Processing as 3 to COS-1 Cell
8 Division Which Performed the Same Processing as 4 to COS-1 Cell

[Translation done.]

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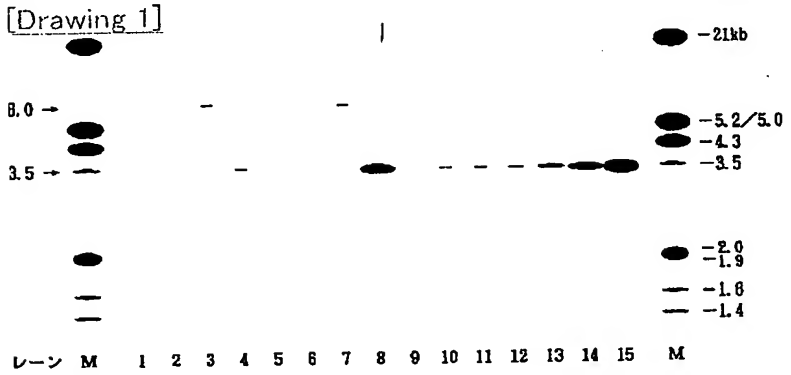
DRAWINGS

[Drawing 1]



[Translation done.]